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Administration of cyclophosphamide changes the immune profile of tumor-bearing mice

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Abstract

Cyclophosphamide (CTX) is often used to create a ‘window’ for more effective therapeutic tumor vaccination. According to a commonly applied protocol, we injected 2 mg CTX intraperitoneally (ip) to mice with small (2-3 mm diameter) or large (5-7, and in one experiment 8-10 mm diameter) subcutaneously (sc) growing tumors from the SW1 clone of the K1735 melanoma, euthanized the mice 4 days later and studied the composition of lymphoid cells by flow cytometry in both spleens and tumors. Administration of CTX increased the percentage of CD3+, CD4+ and CD8+ cells with the increases in tumors being significantly greater than in spleens, and it also increased the percentage of B cells in spleens and tumors. Furthermore, CTX dramatically increased the frequency of tumor-infiltrating CD4 and CD8 cells containing IFN γ , of cells expressing NK1.1, and of cells expressing the dendritic cell markers CD11c, CD80 and CD86, with the greatest increases seen among TIL from mice with small tumors. While CTX decreased the percentage of TIL that expressed CD4 or CD8 together with CD25 and FoxP3 and were therefore considered to be Treg cells, it increased the frequency of TIL that stained for Gr1/CD11b, a marker for MDSC. We conclude that administration of CTX can favorably impact several cell populations that are involved in tumor rejection. However, since CTX has a limited effect on TIL from tumors larger than a few mm diameter and in view of an increased percentage of MDSC among TIL from mice given CTX there is a need for more effective ways to improve tumor vaccination.

Keywords

cyclophosphamide; MDSC; TIL; Treg

Introduction

Tumors selectively express a large variety of shared antigens that can be recognized by T lymphocytes (1) and antibodies (2) as well as antigens that are individually unique for each tumor (3) and may reflect their frequent DNA changes (4) and high mutation rate (5). Nevertheless, the clinical efficacy of therapeutic modalities aiming to increase anti-tumor immunity response via cancer vaccination or adoptive transfer of tumor-reactive T lymphocytes has been modest (6), most likely because of a variety of mechanisms that normally protect against autoimmunity.

Sublethal irradiation of tumor-bearing mice can cause the regression of some small established tumors and facilitate the localization of adoptively transferred tumor-reactive lymphocytes to

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tumors (7-9). Injection of CTX, one of the first approved anti-cancer drugs, can, likewise, facilitate the immunological destruction of small tumors (10) to improve the efficacy of adoptive T cell therapy (11) and of therapeutic vaccination (12-14), although the beneficial effects are rarely detected when the tumors are larger than a few mm in mean diameter (15, 16). The effects of gamma irradiation and CTX on tumor-directed immune responses have been attributed to selective inhibition of tumor-directed suppressive T lymphocytes (10,13, 14).

Like other cytotoxic drugs with anti-cancer activity, CTX has several effects on the immune system (17). To investigate some aspects of this, we applied flow cytometry to characterize tumor-infiltrating lymphoid cell populations (TIL) from mice that had growing tumors from the SW1 clone of the K1735 melanoma, a line that we have studied in the past (18). We recently demonstrated that injection of 2 mg CTX made tumor vaccination, 4 days later, therapeutically efficacious against sc growing SW1 tumors which had a mean diameter of 2-3 mm but not against tumors larger than that (16).

Data reported here indicate that administration of CTX profoundly affects the composition of TIL, that these effects are more pronounced in small tumors and much more dramatic in tumors than in spleens from the tumor-bearing mice. While injection of CTX favors the accumulation of cell types known to be involved in tumor rejection supporting the view (19,20) that immunological effects of cytotoxic anti-cancer drugs may contribute to their efficacy, we also observed an increased accumulation of TIL staining for Gr1CD11b, a marker of myeloid-derived suppressor cells (MDSC).

Materials and Methods

Mice and tumor cells

Six to eight-week old female C3H/HeN mice were purchased (Charles River Laboratories, Wilmington, MA). The SW1C clone of the K1735 melanoma is of C3H/HeN origin (21). The animal facilities are ALAC certified, and our protocols are approved by University of Washington's IACUC Committee.

Animal studies

Mice were transplanted s.c. on both sides of the back, with 10^6 tumor cells. When the tumors were either 2-3mm ("small tumors") or 5-7 mm ("large tumors") in mean diameter, mice in the experimental groups were injected i.p. with cyclophosphamide (CTX; Sigma Aldrich; St. Louis, MO), 2mg/mouse in 0.1 ml PBS, while the control groups got 0.1 ml PBS. Four days later, the mice were euthanized and spleens, axillary and inguinal lymph nodes and tumors were harvested. In order to have sufficient number of TIL to analyze, each pool of small tumors (from CTX treated or control mice) was derived from the bilateral tumors of 5 mice (i.e. 10 tumors/pool). To further investigate the relationship between tumor size and effects of CTX, an additional experiment was performed in which the mice had tumors of 8-10 mm mean diameter when they were injected with CTX.

Preparation of lymphoid cells from spleens and tumors

Spleen cell suspensions were prepared mechanically, passed through a cell strainer and centrifuged at 1,500 rpm for 5 minutes, after which the cell pellet was resuspended in 1 ml RBC lysis buffer (Sigma, St.Louis MO). After incubation at room temperature for 4-5 minutes with occasional shaking, the reaction was stopped by adding 20 ml PBS, and the cells were washed twice with DMEM complete medium and counted.

To obtain tumor-infiltrating lymphoid cells (TIL), pooled tumors were cut into small pieces and placed in 10 ml Hank's Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA) containing 10mg collagenase (grade IV; Sigma, St. Louis MO), 0.01mg hyaluronidase (Sigma, St. Louis MO), and 1mg DNAase (Sigma, St. Louis MO). After 1.5 hours incubation on a rocking platform at 37°C, the pellet was resuspended in DMEM (Invitrogen, Carlsbad, CA) and passed through a sterile cell strainer. The resulting cell suspension was washed once in HBSS, resuspended in 5ml 33% Percoll (in HBSS) and placed in a sterile 15 ml conical tube, which was centrifuged for 20min at 2300 rpm at room temperature. The upper-layer and supernatant was removed completely by vacuum. After washing with DMEM once, the cells were incubated with 1 ml Red Blood Cell lysis buffer (Sigma) for 5 min at room temperature; subsequently, 25 ml sterile PBS was added to stop the lysis. The cells were washed with DMEM twice, each wash followed by centrifugation for 8 min at 1000 rpm at room temperature. We used 33% Percoll gradient to separate tumor cells from lymphoid cells but did not apply different gradients to separate out different cell populations. We are aware that this is a caveat since different lymphoid cell populations may vary with respect to what gradient best suits them.

Antibodies

Mabs to the following antigens were purchased from eBiosciences (San Diego, CA): FoxP3 (FJK-16s), CD86 (GL1), and CD11b (Mac-1) conjugated to FITC; CD3 (145-2C11), CD4 (GK 1.5), CD8 (53-6.7), B220 (RA3-6B2), CD80 (16-10A1), and GR1 (RB6-8C5) conjugated to PE; CD11c (N418), CD25 (PC61.5) and F4/80 (BM8) conjugated to PE-Cy5. Mabs to NK 1.1-PE (PK136), CD19-PE (1D3), INF γ -PE (XMG1.2), was purchased from BD PharMingen (San Jose, CA). A Mab to CD20 (EP459Y6) plus a secondary FITC-labeled Mab was purchased from AbCam (Cambridge, MA). Immunoglobulins with isotypes corresponding to the above Mabs and conjugated to the appropriate fluorochromes, were used to control for nonspecific binding.

Flow cytometry

Single cell suspensions were washed with FACS staining buffer (PBS containing 1% fetal calf serum and 0.09% sodium azide, pH 7.4) and resuspended in 100 μ l FACS staining buffer. To detect surface antigens, cells were stained by incubation with optimal concentrations of the Mabs described above for 30 min at 4°C in 100 μ l of staining solution. After washing twice with staining solution, the cells were fixed with 2% formalin in PBS and analyzed using a Coulter Epics C FACS instrument (BD Biosciences). The lymphocytes were gated using forward- and side-scatter to exclude debris and dead cells, after which 10,000 events were acquired in each assay for analysis. All the data were analyzed by FlowJo software (Treestar Inc., OR, USA). The sum of T, B, NK and CD11c+ cells slightly exceeded 100% in a few experiments. This may have several causes (inclusion of NK+ T cells, CD4/CD8 expressed by some DC, as well as minor errors in analyzing the cells).

Intracellular staining by flow cytometry

For detection of FoxP3 and INF γ , cells were first fixed with IntraPrep Permeabilization reagent (BD Biosciences) according to the manufacture's protocol. Briefly, the cell suspensions were washed with FACS staining buffer and permeabilized with IntraPrep Permeabilization reagent for 20 min at 4°C. Subsequently, the cells were resuspended in FACS staining buffer and incubated for 30 min on ice in the dark with FITC-conjugated anti-Foxp3 or PE-conjugated anti-INF γ Mabs. After staining, the cells were collected, washed twice in 100 μ l FACS buffer and resuspended in 200 μ l FACS buffer. Flow cytometry analysis was performed as described above.

Statistics

Results are expressed as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Student's *t* test, and *p* value ≤ 0.05 was considered to be statistically significant. For multiple comparisons, the two-tailed Student *t* test was used.

RESULTS

Data summarized in Table 1 show that injection of 2 mg CTX i.p. to C3H mice bearing SW1 tumors, followed by euthanasia 4 days later, increased the percentage of cells that stained for CD3, CD4 or CD8 in both spleens and tumors. The effects on TIL were much more pronounced than on lymphoid cells in the spleens with increases ranging between 275-606 % and 19-48%, respectively. There was no significant difference for these cell populations from mice with small (mean diameter 3-4 mm) and large (mean diameter 5-7 mm) tumors when CTX was injected. The number of cells in the spleens decreased with close to 50% when the mice was euthanized 4 days after being injected with 2 mg CTX, while the mean number of TIL decreased with about one third without any significant differences relating to tumor size.

Table 2 summarizes data where we stained lymphoid cells from spleens and tumors with Mabs specific for CD19 or CD20, which are markers for B lymphocytes (22) or for B220 which, in addition to being expressed on B cells also is present on some additional cells, including plasmacytoid dendritic cells (23). The cell populations expressing these markers were increased in both spleens and tumors after injection of CTX with the largest increases among the TIL.

The percentage of TIL that contained IFN γ significantly increased in the CTX groups with the greatest increase for TIL from small tumors, while there was no significant increase among spleen cells (Fig. 1). In a repeat experiment with tumors of 8-10 mm mean diameter when the mice received CTX, the increase of IFN γ positive cells was one third of that in a parallel group of mice with tumors of 5-6 mm diameter. Treatment of mice with CTX increased the fractions of both CD4+ and CD8+ TIL that contained IFN γ about 4-fold and this increase was approximately the same independent of tumor size. CTX did not significantly increase the CD4+ or CD8+ spleen cells that stained for IFN γ .

As shown in Fig. 2, there was a dramatic increase in the number of NK cells in small tumors from the CTX group and a less dramatic, but still significant, increase in NK cells among the TIL from mice with large tumors following treatment with CTX; the difference among NK cells from CTX-treated mice with small versus large tumors was significant (*p*=0.004). This difference was confirmed in a repeat experiment in which the administration of CTX increased the frequency of NK1.1+ cells with 452% in mice with small tumors as compared to 88% in mice whose tumors had 5-6 mm diameter and 38% in mice whose tumors had 8-10 mm diameter when CTX was administered. The percentage of NK cells in spleens was slightly (and statistically significant) higher in tumor-bearing mice that were given CTX with no difference related to tumor size.

Fig 3 demonstrates that CTX increased the fraction of TIL expressing CD11c, CD80 and CD86, which are markers of mature dendritic cells. The increase was 10-20 fold for small and 5-10 fold for large tumors. The difference between percentage of cells in small versus large tumors that expressed CD11c or CD80 was significant (*p*=0.003 and 0.002, respectively), while the difference for cells expressing CD86 was not (*p*=0.123). The percentage of spleen cells expressing CD80 and/or CD86 also increased significantly in the CTX group, although the increase was less than for the TIL. In a repeat experiment, CTX increased the frequency of CD11c+ TIL from mice whose tumors had 2-3 mm mean diameter with 274% as compared to a 91% increase among TIL from mice with tumors of 8-10 mm diameter. The percentage of

CD80+ cells increased with 114% in mice whose tumors had 5-6 mm diameter as compared to 43 % in mice with 8-10 mm tumors, and the corresponding figures for CD86+ cells in these two groups were 28% increase versus 48% decrease.

Table 3 demonstrates that the fraction of CD4 or CD8 positive cells that expressed the Treg cell marker FoxP3 was decreased for both small and large tumors, confirming published studies demonstrating a similar effect of CTX on other tumors (13,14). In contrast, a population expressing GR1/CD11b, which is a marker of MDSC (24), was significantly increased in tumors from the CTX group. In another experiment, 0.9% of TIL from mice with large tumors were CD11b+Gr1+ in the control (PBS) group as compared to 4.1% in the CTX group and the corresponding figures for cells that were CD11b+Gr1+F4/80+ were 0.4 and 1.4, respectively.

All experiments were repeated at least twice with similar findings.

Discussion

We show that administration of 2 mg CTX affects a multitude of cell populations among the TIL from mice bearing the SW1 clone of the K1735 melanoma. In particular, TIL from CTX-treated mice with small tumors contained a dramatically increased percentage of cells expressing CD80, CD86 and CD11c which are markers of mature dendritic cells (25), as well as of NK cells and of cells that contained IFN γ , and a significant, albeit smaller, increase of cells expressing CD3, CD4 or CD8. In addition, the percentage of TIL expressing the B cell markers CD19 and CD20 and of cells expressing B220 was increased. Similar but much less striking differences were seen with spleen cells from mice given CTX. The increase of B cells in tumors and spleens was unexpected in view of published evidence that CTX profoundly decreases the population of B cells in lymphoid tissues (17). However, we have only studied the effects of 2 mg CTX and euthanized the mice 4 days later, the time when tumor vaccination is commonly performed, and expect that the effects of CTX are time-dependent. For example, while there was a relative increase in spleen cells expressing CD3, CD4 or CD8 4 days after administration of 2 mg CTX, there was a decrease of the same cell populations after an additional 4 days (Y. Yang et al, unpublished findings).

In agreement with published findings (13,14), the frequency of CD4+CD25+ TIL that expressed FoxP3, a marker of regulatory T cells (26,27), was decreased in tumors from mice given CTX, as was the percentage of TIL expressing CD8, CD25 and FoxP3. In contrast, there was an increase of TIL that were CD11b+Gr1+, a characteristic of MDSC (24). The increased frequency of these cells may result from stimulation via the higher level IFN γ within tumors from mice that received CTX, since IFN γ is known to up-regulate the population of MDSC (28). CTX has been shown to transiently increase MDSC in non-tumor-bearing animals (29) and a higher level of MDSC has been reported in patients with advanced cancer who received a combination of doxorubicin and CTX (30). This is a concern, and we will investigate the effect of CTX on MDSC further by performing functional assays and by administering drugs reported to have selective effects on MDSC.

The CTX effects were significantly greater on TIL from mice with small (2-3 mm mean diameter) than large (5-7 mm) tumors both with respect to mature DC, NK cells and cells staining for intracellular IFN γ , all of which have shown to be involved in the immunological destruction of SW1 tumors (15,16,18,31). These effects further decreased when the tumors had 8-10 mm diameter, and it is noteworthy that a combination of tumor vaccination with CTX is rarely effective against large tumors (13,15,16). It is not surprising that the effect of CTX decreases with tumor size, since molecules that can inhibit an anti-tumor response are present at high levels in tumors (32), including tumor antigen (33) and TGF β which is made at high levels by SW1 cells (16).

We speculate, as have others (20,21), that many cytotoxic anti-cancer drugs have immunomodulatory effects which contribute to their therapeutic activity. It is likely that better knowledge of these effects, which are probably different for different drugs, can improve cancer therapy.

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Abbreviations

CTX	cyclophosphamide
DC	dendritic cells
IFN	interferon
ip	intraperitoneal
MDSC	myeloid derived suppressor cells
PBS	phosphate buffered saline
sc	subcutaneous
TIL	tumor-infiltrating lymphoid cells
Treg	regulatory T cells

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Fig. 1. Percentage of TIL containing intracellular IFN γ in small and large tumors from mice given 2 mg CTX or PBS (as control). Samples from 5 mice/group were pooled with 2 tumors (bilateral) per mouse. Data are presented in panel A and summarized in panel B. Statistical significance indicated: * $p < 0.05$

Fig. 2.

Percentage of cells expressing NK1.1 in small and large tumors from mice given 2 mg CTX or PBS (as control). Samples from 5 mice/group were pooled with 2 tumors (bilateral) per mouse. Data are presented in panel A and summarized in panel B. Statistical significance indicated: * $p < 0.05$; ** $p < 0.01$

Fig. 3.

Percentage of cells expressing dendritic cell markers in small and large tumors from mice given 2 mg CTX or PBS (as control). Samples from 5 mice/group were pooled with 2 tumors (bilateral) per mouse. Data are presented in panels A and B and summarized in panels C and D. Statistical significance indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 1
Effects of CTX on T cell populations from mice with small or large tumors*

Samples	Markers	Small PBS	Small CTX	P value	Large PBS	Large CTX	P value
Spleen	CD3+	20.9 ± 0.2	24.8 ± 0.3	0.004	17.7 ± 1.1	26.1 ± 0.9	0.013
	CD4+	15.2 ± 0.1	19.9 ± 2.3	0.100	14.3 ± 1.0	18.3 ± 0.2	0.028
	CD8+	6.6 ± 0.3	9.4 ± 0.3	0.012	6.3 ± 0.0	9.7 ± 0.2	0.001
Tumor	CD3+	4.5 ± 1.3	31.7 ± 5.9	0.024	10.2 ± 3.7	38.2 ± 6.6	0.034
	CD4+	1.4 ± 0.0	10.8 ± 0.0	0.000	1.2 ± 0.2	8.2 ± 0.9	0.009
	CD8+	2.6 ± 0.3	18.0 ± 0.3	0.000	3.3 ± 0.2	17.3 ± 0.9	0.002

* Tumors are referred to as small when their mean diameter is 2-3 mm and as large when it is 5-7 mm.

Table 2
Effects of CTX on spleen and tumor B cell populations from mice with small or large tumors*

Samples	Markers	Small PBS	Small CTX	P value	Large PBS	Large CTX	P value
Spleen	CD19+	55.4 ± 1.6	66.5 ± 3.3	0.050	47.1 ± 3.6	62.1 ± 2.0	0.036
	B220+	57.7 ± 1.1	68.2 ± 0.4	0.007	54.7 ± 2.3	64.3 ± 0.2	0.036
	CD20+	11.1 ± 0.9	16.9 ± 1.3	0.035	16.8 ± 0.1	16.7 ± 3.2	0.944
Tumor	CD19+	9.3 ± 1.2	24.7 ± 1.9	0.010	6.6 ± 0.5	35.4 ± 6.7	0.026
	B220+	7.1 ± 0.8	13.1 ± 1.4	0.033	4.6 ± 0.1	21.6 ± 0.6	0.001
	CD20+	0.7 ± 0.0	4.0 ± 0.1	0.001	0.7 ± 0.0	3.6 ± 0.2	0.001

* Tumors are referred to as small when their mean diameter is 2-3 mm and as large when it is 5-7 mm.

Table 3
Effects of CTX on spleen and tumor regulatory T cells and MDSC from mice with small or large tumors*

Markers expressed	Small PBS	Small CTX	P value	Large PBS	Large CTX	P value
CD25+Foxp3+/CD4+	2.9 ± 0.3	1.4 ± 0.2	0.026	3.5 ± 0.3	0.9 ± 0.1	0.007
CD25+Foxp3+/CD8+	1.8 ± 0.1	0.9 ± 0.0	0.000	1.0 ± 0.3	0.4 ± 0.0	0.108
Gr1+ CD11b+	0.1 ± 0.0	2.3 ± 0.5	0.020	0.1 ± 0.0	1.1 ± 0.0	0.001

* Tumors are referred to as small when their mean diameter is 2-3 mm and as large when it is 5-7 mm.